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Effect of Auxin concentration of Callus Induction from *Justicia gendarussa* L. Stem and Leaf Explants

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ABSTRACT:
In the present paper the effect of different concentrations of auxins (0.1 – 3mg/l) on the callus induction and organogenesis from *Justicia gendarussa* L., a member of Acanthaceae was studied. M.S. medium with the concentration of 2, 4 – Dichlorophenoxy acetic acid at 2mg/l induced the highest percentage of callus (83%) and therefore, this medium was found to be suitable for callus induction. 2, 4 – Dichlorophenoxy acetic acid at the concentration of 2mg/l and α - Naphthalene acetic acid at the concentration of 1mg/l induced significant percentage of callus (77% and 83% respectively) in stem and both 2, 4 – Dichlorophenoxy acetic acid and Naphthalene acetic acid at the concentration of 1mg/l induced 67% and 72% of callus respectively in leaf explants. Direct organogenesis (50%) by activating axillary buds from the nodal explants was observed in the medium supplemented with Indole 3 acetic acid at the concentration of 0.5mg/l. Internodal and leaf explants did not show any response. The plantlets obtained were subjected to hardening using sterile sand: soil: manure mixure (1:1:1).

Key words: *Justicia gendarussa*, callus, auxin, organogenesis

INTRODUCTION

For the support of good growth of tissues and organs, it is necessary to provide GR in the nutrient medium. Commonly used GR include auxin, cytokinin and gibberellins. The common auxins are IAA, IBA, 2, 4 - D, 2, 4, 5 - T, NAA etc and that of cytokinins are 6-BA, Kinetin, 2iP, TDZ etc. IBA and IAA when used alone induces root formation whereas, in combination with cytokinin induces shoot formation. 2, 4 – D and 2, 4, 5 – Trichloroacetic acid are considered to be the potent callus inducers. 2, 4 – D is also used in embryogenesis. All the cytokinins used in the culture have their role in cell division and differentiation of adventitious shoots from callus and organs [1].

In general, heterogenous callus cells have the capacity to undergo redifferentiation to give rise to embryos, roots and shoots. Sometimes, the embryonic explants exhibit direct differentiation into root or shoot without the formation of callus. Factors affecting cytodifferentiation include, GR, sucrose, calcium, physical and physiological factors [1]. *Decalepis hamiltonii* leaf explants cultured on Murashige and Skoog (MS) medium supplemented with NAA+ 6- BA induced compact callus while the media supplemented with 2, 4 -D + BA induced embryogenic callus [2]. *In vitro* propagation of *Justicia gendarussa* on M.S. medium supplemented with low concentrations of NAA and 6-BA (1mg/l and 0.1mg/l respectively) was carried out by Agastian et al [3]. A protocol for the direct organogenesis of *Justicia gendarussa* plants were achieved using nodal explants on M.S. medium supplemented with 4.44 µM BA [4, 5]. Different sources mention the medicinal properties of the plant for various health problems such as intermittent fevers, rheumatism, dysuria, fever, carbuncles, jaundice, diarrhea, pains in the head, ear, paralysis, bruises etc and the plant has cooling, anodyne, antispasmodic, carminative, antiperiodic, emetic properties. The dried leaves are used to repel insects from clothing [6-9].

The aqueous leaf extract of *Justicia gendarussa* is known for its antinociceptive activity in rats [10]. One of the methods to harvest the important plant components is through tissue culture methods. It has also been possible to obtain the novel compounds through callus [11]. The method of obtaining novel compounds using *in vitro* technique reduces the pressure on the wild plants. Keeping in mind the wide uses of *Justicia gendarussa* in the treatment of diseases, the present study is aimed at developing the callus and to find out the effect of different types of auxin concentrations on callus induction.

MATERIALS AND METHODS

Plant material

The plant material *Justicia gendarussa* L. (*Vern* name: Tel - Nalla vavili, Tam - Karu nochi) a member of family Acanthaceae (2n=30) were collected from natural forests of Dakshin Kannada Districts, Karnataka, India. The evergreen, shade loving plant is a highly branched shrub grows to 0.8-1.5m height with opposite lanceolate leaves (12.5 X 2.5cm), short petioled with acute purple veins at the base which tapers into rounded apex. The plant has quadrangular stem thickened at and above the nodes and internodes 2-7cm long. The flowers are terminal or axillary, irregular, bisexual, sessile, white with pink or purple spots inside and red at throat and lip [7, 9, 12]. Healthy plant materials such as nodal and internodal regions and leaf samples were collected in different seasons for different trials from the mother plant in the vegetative stage. They were washed under running tap water for 45 min and treated with 1% Bavistine (30min), 70% alcohol (1min) and 0.1% $HgCl_2$ (8min) and washed with sterile distilled water three to four times. The sterilized plant materials were cut into ~ 0.5 -1cm long explants and used for inoculation. The young, mature and highly mature explants from the stem were inoculated separately.

Culture media

Three different types of culture media *viz*: M.S.[13], B5 [14] and Nitsch [15] were used in the present study to find out suitable medium for callus induction. M S medium is a versatile medium with higher nutrient

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supply used for callus induction in most of the plant materials while, B 5 medium is relatively with reduced concentration of nutrients, used for culturing of selective plant materials and Nitsch medium is generally used for haploid culture. Macro elements, microelements and organic supplements of the media were prepared as 20x and 200x stock solutions respectively. As per the requirement, the working media was prepared by diluting the stock solutions and steam sterilized at 121ºC with a pressure of 15psi for 15-20min. The media without any GR served as control media.

Plant GR used in the study

In the present study, three types of auxins were used: *viz*. 2, 4 – D, NAA and IAA. Stock solutions of GR were prepared by dissolving them in ethanol/1N NaOH to prepare the medium with the following working concentrations: 0.1, 0.5, 1, 2 and 3 mg/l. The medium without any GR served as control. The cultures were incubated at 25±2°C with 16hrs of photoperiod using 40 W white fluorescent lights.

OBSERVATIONS

The explants were observed for various details such as development of callus from mature or immature regions of the explants, nature of callus, color / pigmentation of the callus, initiation of roots and shoots, length of roots and shoots etc. after incubating for 25days. For the microscopic observation, the callus cells were squashed on a clean glass slide with a drop of glycerin and observed under the microscope for size, shape and cytodifferentiation processes.

HARDENING OF THE PLANTLET

The plantlets obtained from the nodal explants in the medium containing IAA were carefully removed from the solid medium after one month and transferred to M.S. liquid medium without any GR and incubated in the culture room for one month. Later, these plants were subjected to hardening. For this purpose, sterile soil:sand:manure mixture (1:1:1) was used and the plantlets were gradually exposed to the external environment using polythene covers. The polythene covers with single small holes were kept upside down over the plants and gradually the size of the hole was increased after every two to three days. After two weeks, the polythene cover was removed and the plants were exposed to the open environment. The plants were watered using sterile distilled water for one week and slowly changed to distilled water and tap water.

Altogether, 22 plantlets were transferred from the solid medium to liquid medium and from there to soil:sand :manure mixture as mentioned above. The number of leaves developed and the length of roots were noted.

STATISTICAL ANALYSIS

The statistical analysis of all the data was carried out using Agres (ANOVA package for researchers Ver 7.01, © 1994 Pascal In 4 Software Solutions) following completely randomized design (CRD) with p- test of significance.

RESULTS

Effect of culture media

There was no significant variation in the growing callus between the three types of nutrient media used in the present study (Table 1). M.S. medium with the concentration of 2, 4 –D induced the highest percentage of callus (83%). Therefore, for all further experiments the most common culture medium – M.S. medium was selected. The mature explants showed successful callus initiation, young explants did not respond well with the culture conditions (Figure 1a). However, the highly matured explants responded positively both in control and in the media containing auxins. The callus initiation was observed from the cut ends after a week of incubation and spread to the remaining regions of the explants.

Effect of 2, 4 – D on *J. gendarussa* stem and leaf

The calli obtained at different concentrations of 2, 4 – D were similar in terms of morphology but varied with regard to the percentage of calli obtained in each of the concentrations. 2, 4 – D at the concentrations of 1mg/l and 2 mg/l for stem explants (Table 2) and 0.5 mg/l and 1 mg/l for leaf explants (Table 3) were found to be ideal as the percentage of callus obtained in these concentrations were significantly higher ($p = <0.05$) than the rest of the concentrations. The callus obtained was initially creamish in color and as the age increased, the color changed to pale yellowish and pinkish (Figures 1b & c). This color change was more prominent in stem explants than leaf explants. The callus was too friable and watery in nature and as a result, sub culturing of this friable callus was very difficult. Green callus was also observed but, the amount of green calli were less in the medium supplemented with 2, 4 – D.

Effect of NAA on *J. gendarussa* stem and leaf

Similar to that of 2, 4 – D, there was no morphological variation in the callus obtained from the different concentrations of NAA. The callus induction was significantly higher ($p = <0.05$) at the concentrations of NAA, 1 mg/l and 2 mg/l for stem explants (Table 2) and 0.5mg/l, 1mg/l and 2mg/l for leaf explants (Table 3) compared to the other concentrations. When NAA was used in the medium, a small amount of callus was developed after a week of incubation followed by root emergence. The root development was significant ($p = <0.05$) at the concentrations of 2mg/l for stem explants (Table 2) and 1mg/l and 2mg/l for leaf explants (Table 3). The morphology of the callus was highly differing from that of 2, 4 – D callus with yellowish and greenish in appearance. The cultures away from the light were more yellowish and those were near to the light were greenish in color. After 15d of incubation, there was a large amount of thin and long roots emerging out of the callus both from stem and leaf

explants (Figures 1d & e). The root development was either from the callus or directly from the explant. Some of the nodal explants showed the development of 1 or 2 shoots from the axil and more roots from the basal end.

Effect of IAA on *J. gendarussa* stem and leaf

The callus induction in both stem and leaf explants was insignificant (p = >0.05) in the medium supplemented with IAA when compared to other two GR (Table 2 & 3). The callus was yellowish and greenish in color and hard in texture (Figures 1f & g). The stem explants showed the development of significant percentage (p = <0.05) of roots (57%) and plantlets (50%) at the concentration of 0.5mg/l and this response was at a high rate than callus induction rate. Whereas, the leaf explants were not responding well with medium supplemented with IAA. The nodal explants showed the development of 2-3 shoots from the axil and roots from the basal region. The roots formed were not in direct contact with those of shoots in majority of the explants.

Microscopic observations

The microscopic observations of callus obtained in medium with 2, 4 –D revealed the presence of small round cells with or without chlorophyll, slightly elongated cells (Figures 2a & b), cells were showing xylogenesis (Figures 2c & d). Chlorophyll pigment was more in leaf callus than in stem callus. The cells of the leaf callus were more elongated when compared to the cells of stem callus. Microscopic observations of callus obtained in the medium with NAA revealed the presence of some hockey stick shaped cells and curved cells in the mass of normal callus cells (Figures 2e & f). The root hairs were prominently seen in stem callus whereas, a few elongated cells were seen in leaf callus, which might be the future root hairs. In medium supplemented with IAA, the callus cells were small, round or curved with or without chlorophyll (Figure 2g).

Hardening of the plantlet

In solid medium, the plantlets with a shoot length of 2 - 2.5cm and root length of 2 - 3cm had 6-8 leaves (Figure 3a). 22 plantlets were transferred from solid to liquid medium and all of them survived. The plantlets in the liquid medium had a shoot length of 4 - 5cm and root length of 3 - 4 cm with 8 - 10 leaves (Figure 3b), which were later transferred to polythene bags (Figure 3c).

Table 1: Effect of different types of nutrient media on thegrowth of callus in *J. gendarussa*

Growth regulator		Callus induction in different media					
		MS		B 5		N	
		(Mean ± SE)▪	%	(Mean ± SE)▪	%	(Mean ± SE)▪	%
2, 4 – D	0.5mg/l	5.3 ± 1.16	53	5.3 ± 0.58	53	5.3 ± 0.58	53
	1mg/l	6.7 ± 0.58	67	6.3 ± 0.58	67	6.3 ± 0.58	63
	2mg/l	8.3 ± 0.58*	83	8.0 ± 1.00*	80	7.7 ± 1.53*	77

Table 1 Legend:

▪ The data are average of 3 trials of 10 tubes each CD (0.05) for media =0.85240, conc =0.85240, media + conc = 1.47640

* p = <0.05

Table 2: Effect of auxins on *J. gendarussa* stem explant

Growth regulator– in mg/l		Callus induction		Root induction		Plantlet induction	
		(Mean ± SE)▪	% of callus induction	(Mean ± SE)▪	% of callus induction	(Mean ± SE)▪	% of callus induction
2, 4- D	0.1	4.3 ± 1.53	43	0.0 ± 0.00	0	0.0 ± 0.00	0
	0.5	5.3 ± 1.16	53	0.0 ± 0.00	0	0.0 ± 0.00	0
	1	6.7 ± 0.58 *	67	0.0 ± 0.00	0	0.0 ± 0.00	0
	2	7.7 ± 1.53 *	77	0.0 ± 0.00	0	0.0 ± 0.00	0
	3	4.3 ± 1.16	43	0.0 ± 0.00	0	0.0 ± 0.00	0
NAA	0.1	3.0 ± 1.73	30	1.7 ± 1.53	17	0.0 ± 0.00	0
	0.5	3.7 ± 2.52	37	3.0 ± 0.00	30	0.0 ± 0.00	0
	1	8.3 ± 1.16 *	83	4.3 ± 1.53	43	0.0 ± 0.00	0
	2	6.7 ± 3.22 *	67	7.3 ± 0.58 #	73	0.0 ± 0.00	0
	3	1.3 ± 1.53	13	2.3 ± 2.08	23	1.0 ±1.73	10
IAA	0.1	3.0 ± 2.00	30	4.7 ± 0.58	47	3.3 ± 0.58	33
	0.5	1.0 ± 1.00	10	5.7 ± 0.58 #	57	5.0 ± 1.00 ♦	50
	1	1.0 ± 1.00	10	3.0 ± 1.73	30	2.0 ± 1.00	20
	2	2.7 ± 0.58	27	1.7 ±1.53	17	1.3 ± 1.16	13
	3	2.7 ± 2.31	27	3.0 ± 2.65	30	1.0 ± 1.00	10

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Table 2 Legend:

▪ The data are average of 3 trials of 10 tubes each

For callus induction: CD (0.05) for growth regulator = 1.25773, conc = 1.62372, growth regulator + conc = 2.81237

* p = <0.05

For root induction: CD (0.05) for growth regulator =0.90995, conc =1.17475, growth regulator + conc = 2.03472

p = <0.05

For plantlet: CD (0.05) for growth regulator =0.53315, conc =0.68829, growth regulator + conc = 1.19215

♦ p = <0.05

Table 3: Effect of auxins on *J. gendarussa* leaf explant

Growth regulator– in mg/l		Callus induction		Root induction	
		(Mean ± SE)▪	%	(Mean ± SE)▪	%
2, 4- D	0.1	6.0 ± 1.00	60	0.0 ± 0.00	00
	0.5	5.3 ± 1.16 *	53	0.0 ± 0.00	00
	1	6.7 ± 0.58 *	67	0.0 ± 0.00	00
	2	5.0 ± 2.00	50	0.0 ± 0.00	00
	3	2.3 ± 1.16	23	0.0 ± 0.00	00
NAA	0.1	5.0 ± 2.00	50	0.0 ± 0.00	00
	0.5	7.3 ± 1.53 *	73	4.3 ± 1.16	43
	1	7.7 ± 1.53 *	77	7.0 ± 1.00#	70
	2	6.7 ± 2.52 *	67	8.0 ± 1.00#	80
	3	6.0 ± 1.00	60	6.0 ± 1.00	60
IAA	0.1	0.0 ± 0.00	00	0.0 ± 0.00	00
	0.5	0.7 ± 1.16	07	0.0 ± 0.00	00
	1	0.7 ± 1.16	07	0.0 ± 0.00	00
	2	5.3 ± 1.53	53	0.0 ± 0.00	00
	3	4.3 ± 0.58	43	0.0 ± 0.00	00

Table 3 Legend:

▪ The data are average of 3 trials of 10 tubes each

For callus induction: CD (0.05) for growth regulator =0.99432, conc =1.28366, growth regulator + conc = 2.22337

* p = <0.05

For root induction: CD (0.05) for growth regulator =0.40082, conc =0.51746, growth regulator + conc = 0.89627

p = <0.05

DISCUSSION:

M. S. medium was used for callus induction in varieties of plants such as *Solarium melongena* L [16], Okra [17], Summer squash [18], *Ficus Anastasia* [19] including *J. gendarussa* [4, 5]. In the present study also, explants of *J. gendarussa* responded well with M. S. medium.

In the present study, 2, 4 – D at the concentration of 1 and 2 mg/l for stem and 0.5 and 1 mg/l for leaf induced friable callus. Bushrabi et al [4] made similar observations in the same plant. In *Heavea brasiliensis* Mull. Arg., 3, 4 –D induced the formation of friable callus and the friability of the callus was influenced by sucrose, calcium and some macronutrients other than GR [20]. An effective protocol for complete plant regeneration in *Ocimum basilicum* L. through somatic embryogenesis from leaf explants using 2, 4 – D at the concentration of 1 mg/l in M.S. medium was developed by Gopi and Ponmurugan [21]. Friable, shiny-white and watery callus was obtained from leaf explants of *Hordeum vulgare* (cv. Karan 92) when cultured on M.S. basal medium supplemented with 2**,** 4-D [22].

The hypocotyl explants of *Solarium melongena* L., when cultured in medium with varied concentrations of NAA induced various responses. Induction of callus was observed at 0.8 mg/l concentration. NAA at lower concentration (0.016mg/l) induced the formation of callus, adventitious roots and shoots. Embryoid formation was observed at higher concentration of NAA i.e. at 8mg/l. shoot, root etc [16].

In the present study, IAA did not induce significant percentage of callus. However, IAA induced the development of roots and plantlets at the concentration of 0.5mg/l. The shoot induction in response to plant auxin alone has not been reported so far. There is a report indicating the influence of bacterial IAA on adventitious shoot formation in *Brassica oleracea* L [23]. The IAA was used to induce roots in microcuttings of strawberry tree (*Arbutus unedo* L.) [24], Oleander (*Nerium oleander* L.) [25] and *Capparis spinosa* [26]. Both NAA and IAA were reported to be used as rooting hormones in various experiments, *viz*. in Okra [17], *Curculigo orchioides* Gaertn [27], summer squash [18], *Ficus Anastasia* [19], *Malus* 'Jork 9'[28].

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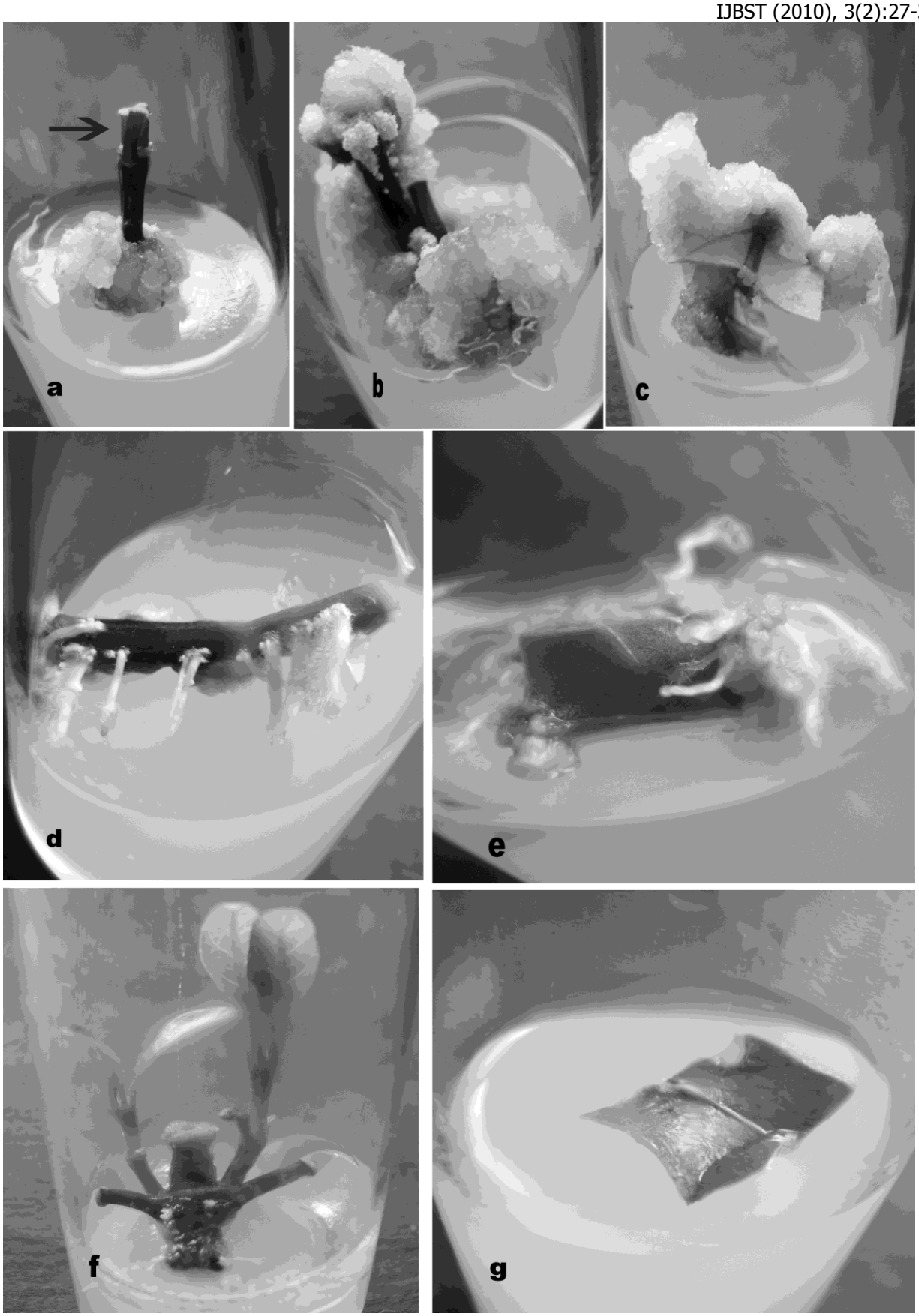

Fig 1 Response of explants to *in vitro* condition. (a) mature part of the explant showing callus and immature part of the explant without any response, where → indicates immature region (b, c) effect of 2, 4 – d on stem and leaf (d, e) effect of NAA on stem and leaf (f, g) effect of IAA on stem and leaf

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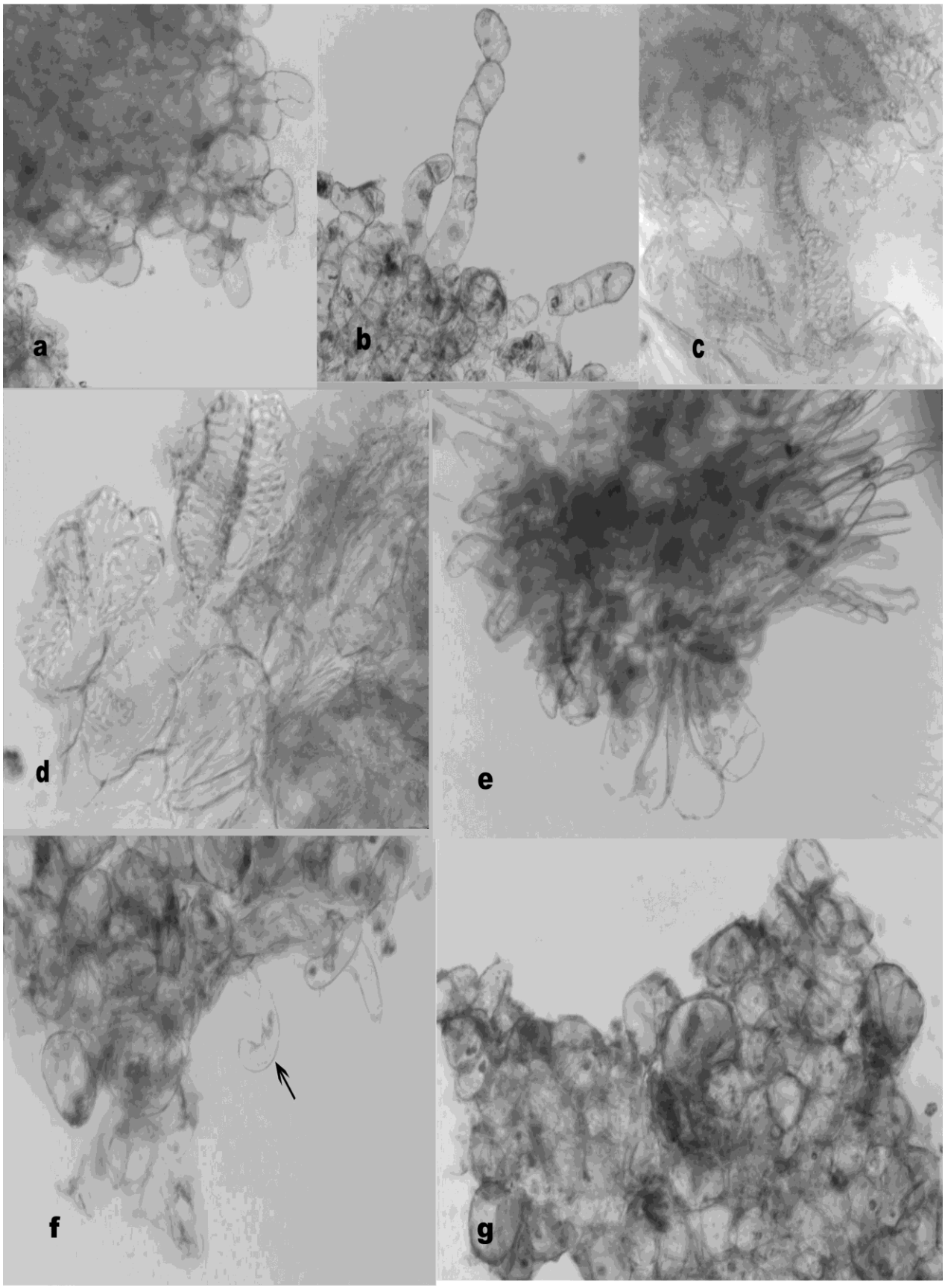

Fig 2 Microscopic observation of callus (a) stem callus obtained using 2, 4 – D (b) leaf callus obtained using 2, 4 – D (c) xylogenesis in stem callus (d) xylogenesis in leaf callus (e) stem callus obtained using NAA (f) leaf callus obtained using NAA, where → indicates hockey stick shaped cells (g) stem callus obtained using IAA

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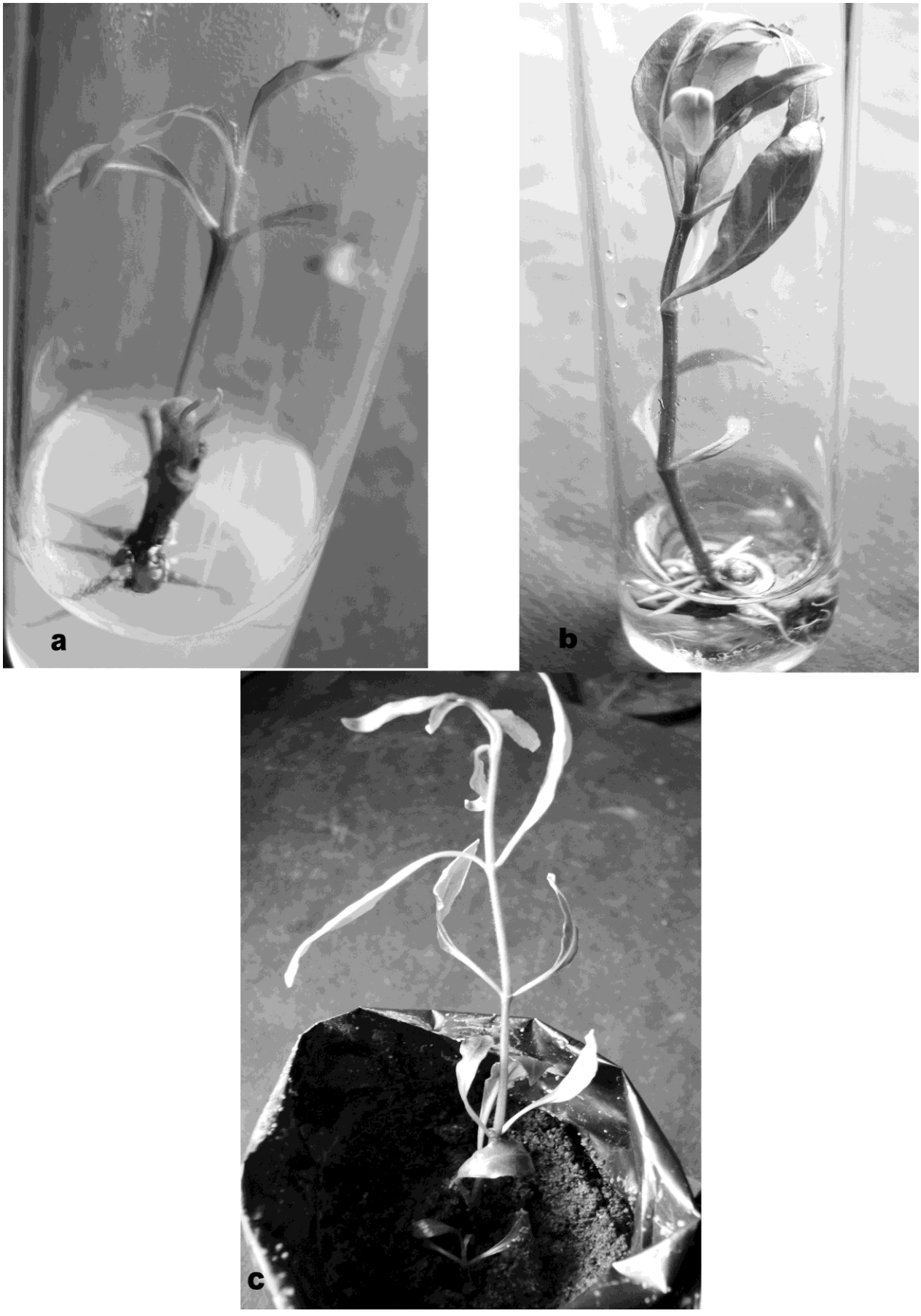

Fig 3 Hardening (a) explant showing axillary shoot induction (b) subcultured axillary shoot in liquid M. S. medium (c) plant transferred to polythene bags

Kato [29] induced callus from *Pteris vittata* roots and observed the presence of mature parenchymatous cells at the centre with meristematic cells at the periphery. The presence of elongated tubular cells, giant cells, curved free cells, spiral configurations, rope –like twisted cells in the callus mass and starch grains in large amount and a few oil droplets were observed by him in the medium containing 2, 4 - D. The reduced amount of chloroplast production with rapid division of callus was observed in *Eckloniopsis radicosa* (Kjellman) by Notoya [30].

Xylogenesis was observed only in 2, 4 - D derived callus in the present study. Xylogenesis, a type of cytodifferentiation reported in *Zinnia elegans* [31], Lactuca [32], rice [33] in which the cells undergo cytoquiscence and cytosenescence and is mainly associated with the redifferentiation of tracheary elements. The process is affected by various factors such as phytohormones, sugars, light, temperature etc. which may or may not be involved in plantlet regeneration from the callus [1].

CONCLUSION

M. S. medium was found to be suitable for callus induction in *J. gendarussa*. Callus was induced from the stem explants at the concentrations of 2mg/l of 2, 4-D and 1mg/l NAA and from the leaf explants at the concentrations of 1 mg/l of both the GR. IAA was involved in the regeneration of plantlets through direct organogenesis by activating axillary buds from the nodal explants.

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Abbreviations:

GR: Growth Regulators
2, 4: D: 2, 4- Dichlorophenoxy acetic acid
NAA: α - Naphthalene acetic acid
IAA: Indole 3 acetic acid
IBA: Indole 3 – Butyric Acid
BA: 6- Benzyl Adenine
TDZ: Thidiazuron
2 iP: n-[2-isopentenyl] adenine
B5: Gamborg's B5 medium
CD: Critical Difference
SE: Standard error